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Molecular Cloning of a Human MafF Homologue, Which Specifically Binds to the Oxytocin Receptor Gene in Term Myometrium

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The US-2 DNA-binding element (ggaatgattactcagctaga) in the promoter of the human oxytocin receptor (OTR) gene has been shown to bind specifically nuclear proteins from human myometrium at parturition. To elucidate the molecular mechanisms involved in OTR gene upregulation at term, the US-2 element was used in a yeast one-hybrid system to screen a cDNA library derived from term human myometrium. Positive clones were further screened by electrophoretic mobility shift assay for their ability to bind the human OTR gene promoter, containing the US-2 motif. A 2.3-kb full-length cDNA encoding a human homologue of chicken MafF (hMafF) was isolated. hMafF represents an 18-kDa protein and contains an extended leucine zipper structure, but lacks a transactivation domain. Furthermore, Northern hybridization showed strong hMafF mRNA expression in the kidney and in term myometrium only, but not in nonpregnant myometrium. The hMafF protein is also preferentially expressed in term myometrium, as shown by specific binding to the OTR promoter. The highly specific binding of hMafF to the US-2 motif in the human OTR gene, together with its pattern of expression, supports a role for hMafF in OTR gene upregulation at term.

Parturition consists of multistage biochemical and biophysical reactions, which ultimately result in the uterine myometrium changing dramatically from a quiescent state to one of active contraction. The molecular mechanisms, which drive this spontaneous change, are still largely unknown. As part of this process, the oxytocin (OT)-oxytocin receptor (OTR) system appears to play a crucial role, with a dramatic upregulation of OT-binding in the human myometrium at term, due to an increase in OTR gene transcription of more than 300-fold after the onset of labor, compared with the level in non-pregnant myometrium (1). The molecular mechanisms underlying the transactivation of the OTR gene, and hence regulating the parturition cascade, are largely unknown. While estrogen is implicated in OTR gene upregulation *in vivo* (2), only weak (rat; 3) or no (human and bovine; 4) influence of estrogen can be shown in transfected cell systems, making it unlikely that direct steroid action can explain the upregulation of the OTR gene.

In an alternative approach to investigate the regulation of the OTR gene, we have compared the expression patterns of nuclear DNA-binding proteins from upregulated (term myometrium) and downregulated (non-pregnant myometrium) tissues interacting with the upstream region of the gene. Using such differential display electrophoretic mobility shift assays (EMSA), we could identify two novel protein-binding elements associated with OTR upregulation (5) and downregulation (6), respectively. We have now identified a third element, US-2 [ggaatgattactcagctaga; corresponding to nucleotides −1433 to −1414 of the human OTR gene (7)], whose occupancy correlates with the upregulation of the OTR gene. Here we report the cloning, using a yeast one-hybrid system, and characterization of a novel DNA-binding protein, which specifically binds the US-2 element in the human term myometrium. This new factor appears to be the human counterpart of chicken MafF, and its specificity of expression makes it a good candidate for an enhancer in the upregulation of the OTR gene at parturition.

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MATERIALS AND METHODS

Tissue preparation. Human non-pregnant myometrium and myometrium of 14 weeks gestation were obtained at hysterectomy. Term myometrium was obtained at hysterectomy due to uterine rupture accompanying vaginal delivery. Placenta and choriodecidual membranes was obtained at elective cesarean section at term. Spleen was obtained at splenectomy; liver and the kidney samples were postmortem. Tissues were rapidly dissected, rinsed with ice-cold saline, snap-frozen in liquid nitrogen and then stored at −80°C until use. All patients (or the family) had provided informed consent and the Helsinki convention was observed in all cases.

Construction of cDNA library. Total RNA and poly(A)$^+$ RNA from term myometrium was prepared as described by Okayama *et al.* (8). Five micrograms of poly(A)$^+$ RNA was converted to cDNA using the SuperScript Plasmid system (for directional library using oligo(dT)-*Not*I/*Sal*I primer; Gibco BRL) and SuperScript Choice system (for random library using random hexamers, Gibco BRL). Size-fractionated (>500 bp) double-stranded cDNA was ligated into the pGAD424-*Sal*I/*Not*I vector (a gift of Dr. B. Gellerson, Hamburg) for the directional library or into pGAD424 (Clontech) for the randomly primed library. Both libraries were transfected into DH5α elctrocompetent cells and indicated complexities of 7.7×10^6 and 6.6×10^6 independent clones, respectively. These libraries were expanded, and plasmid DNA was recovered by ion exchange chromatography (JETStar; Genomed).

Yeast one-hybrid system. The MATCHMAKER One-Hybrid system (Clontech) was used for yeast one-hybrid screening (9). The *cis*-element, US-2, in the 5′-flanking region of the human OTR gene (7), was identified by serial screening of differential-display EMSA, comparing nuclear proteins from term myometrium with those from non-pregnant myometrium (5), followed by methylation interference footprinting (data not shown). One micrograms of sense and antisense strands of the US-2A oligonucleotide (Table 1) was annealed, concatemerized, and the trimeric concatemer purified and subcloned into *Bam*HI-digested pBluescript II (Stratagene) to confirm the sequence. This plasmid was digested with *Sma*I or *Xba*I, the *Xba*I site being polished by a Klenow fill-in reaction, and then the US-2 trimers ($(US\text{-}2)_3$) subsequently excised by *Xba*I or *Eco*RI, respectively. The *Eco*RI–*Xba*I digested pHISi-1 (Clontech), *Eco*RI-blunt $(US\text{-}2)_3$, and *Sma*I–*Xba*I $(US\text{-}2)_3$ fragments were ligated together; the correct resulting plasmid DNA, with a hexameric *cis*-element, was then checked by sequencing and purified ($(US\text{-}2)_6$-pHISi-1). Two micrograms of XhoI-digested $(US\text{-}2)_6$-pHISi-1 was then introduced into Saccharomyces cerevisiae YM4271 by the polyethylene glycol (PEG)/lithium acetate (LiAc) method (10), and plated onto SD (synthetic dropout)/-His plates, to establish the strain $(US\text{-}2)_6$YM4271.

Fifty micrograms of plasmids derived from the directional or random library was introduced into the $(US\text{-}2)_6$YM4271 strain by the PEG/LiAc method, plated onto SD/-His/-Leu/+15 mM 3-amino-1,2,4-triazol (3AT; Sigma–Aldrich), and incubated at 30°C for 5 days. 6.0×10^6 and 9.1×10^6 clones deriving from the two libraries respectively, were screened. Plasmids with cDNA inserts, derived from grown colonies, were rescued in *E. coli* (DH 5α) and sequenced using pGAD424 specific primers (Clontech).

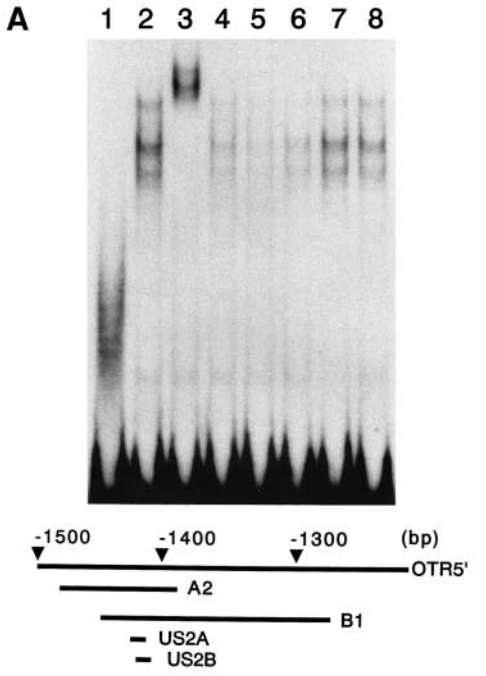
Electrophoretic mobility shift assay (EMSA) screening. After a sequence database search, cDNA inserts with both novel and nuclear protein-related sequences were excised by *Hin*dIII digestion as a cassette together with the GAL4AD sequence, and introduced into the pRcCMV vector (Invitrogen). The correct orientation was checked by PCR, and the GAL4AD-cDNA encoded fusion protein synthesized with the TnT T7-coupled reticulocyte lysate system (Promega). As probe for the EMSA screening, the PCR-derived fragment B1-1 [nucleotides −1466 to −1375 bp (7)], was subcloned into pGEM-T (Promega), excised and labeled with [α^{32}P]dCTP. Two microliters of the TnT product and 1×10^4 cpm of labeled probe was incubated and EMSA was performed as described (5) at 4°C. For supershift experiment, 1 μl of anti-GAL4-TA monoclonal antibody (Santa Cruz) was preincubated with the binding reaction for 30 min at 22°C, then incubated with B1-1 probe for a further 30 min. The EMSA gel was dried and exposed to X-ray film at −80°C.

TABLE 1

Oligonucleotide and PCR Primer Sequences Used for Probe Generation and RACE Reactions

Double-strand oligonucleotides		
US-2A	5′gatcGGAATGATTACTCAGCTAGA	
	CCTTACTAATGAGTCGATCTctag5′	
US-2B	5′gatcGATTACTCAGCTAGAACCCT	
	CTAATGAGTCGATCTTGGGActag5′	
PCR primers		
B1-1	Sense: 5′TTTTCGAGGCCGATAGGTAC3′	
	Antisense: 5′GGACTGGGTTTCCTTAAT3′	
43mer	Sense: 5′CAGCCCCCATTCTGGAATGA3′	
	Antisense: 5′ATCCCAGGGTTCTAGCTGAG3′	
5′-RACE PCR		
AS1:	5′GACTTCTGCTTCTGCAGC3′	95°C 5 min
AS2:	5′GCTTCTGGGCCCGCTTGG3′	95°C 30 s
S1:	5′CGGACGAGGCGCTGATGG3′	*X*°C 30 s
S2:	5′ATCCCCTATCCAGCAAAGC3′	72°C 60 s
(*X*; 65, 63, 61, 59, 57°C; 2 cycles each and 55°C for 25 cycles)		
3′-RACE PCR		
AS(dT):	5′GACTCGAGTCGACATCGATTTTTTTTTTTTTTT3′	
AS1:	5′GACTCGAGTCGACATCG3′	
S1:	5′CACTGTTTATTTATTGCACG3′	
(*X*; 60, 58, 56, 54, 52°C; 2 cycles each and 50°C for 25 cycles)		

Sequencing, 5′- and 3′-RACE, and molecular weight determination. The sequences of the three independent clones whose encoded proteins bound the B1-1 fragment represented partially overlapping fragments of the same gene product. The longest of these clones (#1481) was excised using *Eco*RI, subcloned into pBluescript II (pBS-#1481) and sequenced. To complete this sequence, 5′-RACE was then performed according to Maruyama *et al.* (11). Two micrograms of poly(A)$^+$ RNA from term myometrium was reverse transcribed (Superscript II, GibcoBRL) using a gene specific primer (5′-AS1). The first strand cDNA was self-ligated overnight by T4 RNA ligase (New England Biolabs) and second strand cDNA synthesized from the 5′-S1 primer. This circularized cDNA was used as template in an inverse PCR with the 5′-S2 and the 5′-AS2 primers, together with 1 M betaine (12), *Taq* DNA polymerase (Gibco BRL) and the recommended buffer (Gibco BRL). For 3′-RACE, 2 μg of poly(A)$^+$ RNA from term myometrium was reverse transcribed using the 3′-ASdT primer and the first strand cDNA subjected to a PCR with the 3′-S and the 3′-AS primers. All primer sequences and PCR conditions are listed in Table 1. The PCR product was purified, ligated into pGEM-T vector and sequenced. The molecular weight of the cloned protein was estimated from the TnT product of the plasmid pRcCMV-hMafF after radiolabeling with [^{35}S]methionine (Amersham–Pharmacia). This plasmid was constructed from the *Kpn*I–*Hin*dIII insert of the clone pBS-#1481, which includes the complete open reading frame. The protein product was analyzed by 12% SDS–PAGE using appropriate size markers (broad range; Bio-Rad), and exposed to X-ray film.



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1     GACTGCGGCTCAGAGGCGGAGGGGAGACTGACCGGAGCGCGGATCGGGACAGCGGCCGGGACAGC
66    GGCGAGACGCGCGTGTGTGAGCGCGCCGGACCAAGCGGGCCCAGAAGCGGGTCTGCAGCCCAGAGGGCACCTTCTGCAAAC
147   ATGTCTGTGGATCCCCTATCCAGCAAAGCTCTAAAGATCAAGCGAGAGCTGAGCGAGAACACGCCGCACCTGTCGGACGAG
  1    M  S  V  D  P  L  S  S  K  A  L  K  I  K  R  E  L  S  E  N  T  P  H  L  S  D  E
228   GCGCTGATGGGGCTGTCGGTGCGCGAGCTGAACCGGCATCTGCGCGGGCTCTCCGCCGAGGAGGTGACACGGCTCAAGCAG
 28    A  L  M  G  L  S  V  R  E  L  N  R  H  L  R  G  L  S  A  E  E  V  T  R  L  K  Q
309   CGGCGCCGCACACTCAAAAACCGTGGCTACGCCGCCAGCTGCCGCGTGAAGCGCGTGTGCCAGAAGGAGGAGCTGCAGAAG
 55    R  R  R  T  L  K  N  R  G  Y  A  A  S  C  R  V  K  R  V  C  Q  K  E  E  L  Q  K
390   CAGAAGTCGGAGCTGGAGCGCGAGGTGGACAAGCTGGCGCGCGAGAACGCCGCCATGCGCCTGGAGCTCGACGCGCTGCGC
 82    Q  K  S  E  L  E  R  E  V  D  K  L  A  R  E  N  A  A  M  R  L  E  L  D  A  L  R
471   GGCAAGTGCGAGGCGCTGCAGGCGTTCGCGCGCTCCGTGGCCGCCGCCCGCGGGCCCGCCACGCTCGTGGCGCCGGCCAGC
109    G  K  C  E  A  L  Q  A  F  A  R  S  V  A  A  A  R  G  P  A  T  L  V  A  P  A  S
552   GTCATCACCATCGTCAAGTCCACCCCGGGCTCGGGGTCTGGCCCCGCCCACGGCCCGGACCCCGCCCACGGCCCGGCCTCC
136    V  I  T  I  V  K  S  T  P  G  S  G  S  G  P  A  H  G  P  D  P  A  H  G  P  A  S
633   TGCTCCTAGTGCCCGCCCCCGCCATGCCTCAGCCACGCCCCTCCGGCCTCAGCTCCCTCCCCAAAGTGCCTGAGCGCCGCC
163    C  S  *
714   TCTGTGCCCAGGTCCCATTTCTCTGCAGCACTGGCCCCTTGGTGCACACACATTCCCTTCGTGGGCCCTGTCTTCCTCTTG
795   CAGCCCACCAAACTGGGACCGAATGACCCTGGGAAGGGGAACTTGGGTAGGTTGGGGATGGGGCAGAGGTCTGGATCTGGG
876   ATCGCCCTTGGCTGAAAGTTTAGCCTTTTTAGATTGAGAGATACAGAGCCGGCTTAGAGAACAGCTGTTGGGGGAGAAGAG
957   GGCACCCCTCATCTTGGAAACTGCTCTTATTGTGCCAATATGCCCTCCAAACCCTCCCAGGATTCAAAGCTAGGTTTGGCT
1038  GTCTGTGACTTACGGGACCGTCCTGCTGAGAAATTGCACTGAAGAGATGCCCCCACCTCTGGTTGGGCCTGGGGGTGCCTG
1119  GCCTTCCGAAACTAAAAGAGTGGGTGGGAAGACTAGTGAAACCCAGTTCACGGATGGGGAAACAGGCCTGAGGTCACATTT
1200  CACTTAGTGGTTGTGTTGGGACCAAAACCTGGGTGTCCTCACTGCTGCCCTGAGTCCAGCCATGGTTTTCAGGGGGACAGT
1281  GGACAGGGACTCAGAAATGTGGTGGGAGGGCCTCCCTGGCTTGGGAGACCGCTCTCTGCAAGGGAGGGGGAGAGAAGCAGA
1362  GGGAGAGAGAAGGTGACACGGATGGAAGAGTGGGAAGGAGCTGGCCTGGCTCAGCCCTAGGCTGTCCCTGCAGCCAGGGTG
1443  TCCGGGGGCTGGCCAGTCAGAGAAAGGGGGCCATGGACTGCTGTGGCAAATAGGGAGACAAGGAGACAGACCCTGCAGTCC
1524  TACTACAGTCTGGAGTGGGGTCCTAAGAAGAAGGGTCCCACCTCAACCCCTGTCAGTGTCCACTGTGGGGTGGGGGCTGAC
1605  CCCTGCCTTTGATTGTCATTCTCCTGGGAAGCCCAGTCTCAGTCCCTCCCCCAACACTGTCCACACTGCCCCTCCCCACTG
1686  TTTATTTATTGCACGGATCTAAGTTATTCTCCCCAGCCAGAGCCCGAGCTCCTGCTCCCTGGGAAAAGTGGCGTATGGCCC
1767  TGAGCTGGGCTTTATATTTTATATCTGCAAATAAATCACATTTTATCTTATATTTAGGGAAAGCCGGAGAGCAACAACAAA
1848  AAATGTTTAAGCCGGCGCCGGTGGCTCACATCTGTAATCCCAGCACTTTGGGAGTCCAAGGAGGGGGATCGCTTGAGTCCA
1929  GGAGTTTGAGACCAGCCTGGACAACATGGTGAAACCCCGTCTCTACAAAAAATACAAAAATTAGCCATGCATGGTGGCTCA
2010  TGCCTGTAGTCCCAGCTACTTGGGAGGCTGAGGCAGGAGGATCACTTAAGCCCAGAAGGCAGAGGTTGTAGTGAGCTGAGA
2091  TCGCACCACTGCACTCCAGCCTGGGCAACATAGCAAAATCCTGTCTCAAAAAAAAAGTTAAAAAATATTGCCCGGCTCCTA
2172  GAATTTATTTATTTCCTGACTTACAGCAAGCGAGTTATCGTCTTCTGTATTTTGTAGACTTTCTAAATAAAGTCAAATTCT
2253  TTCTTTTTCCACAGAAGAAAAAAAAAAAAAAAAAAAAA
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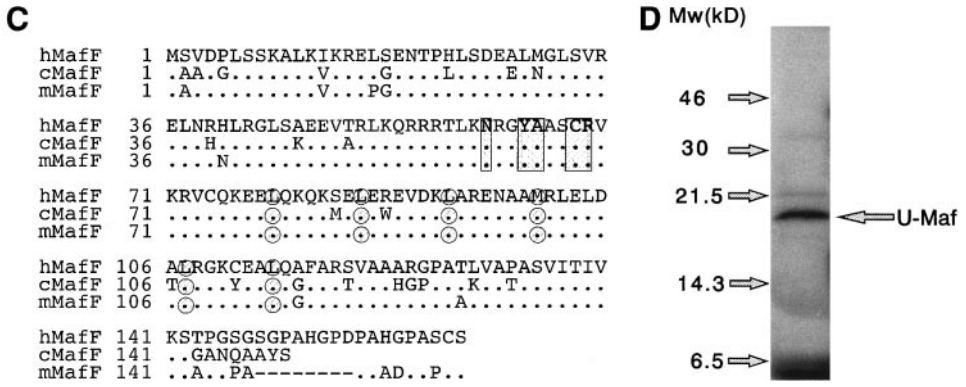

FIG. 1—*Continued*

RNA blot hybridization and comparative EMSA analysis. RNA from various human tissues, as indicated, was prepared using the acid guanidinium-phenol–chloroform method as described (13). Twenty micrograms of total RNA from each tissue was separated by electrophoresis in 2.2 M formaldehyde/1% agarose gels with Mops buffer, transferred onto a Biodyne A membrane (Pall) in 20× SSC, and then hybridized in Quickhyb hybridization solution (Stratagene) at 68°C for 2 h with the [α^{32}P]dCTP labeled 430-bp *Sma*I–*Bam*HI fragment (Megaprime DNA labeling system, Amersham–Pharmacia) digested from pBS-#1481. The membrane was washed in 0.2× SSC–0.1% SDS for 40 min at 65°C and exposed to X-ray film at −80°C. The membrane was reprobed with an [α^{32}P]dCTP-labeled cDNA for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as internal control.

Nuclear protein was purified from different tissues according to the method of Deryckere and Gannon (14) and aliquots snap-frozen in liquid nitrogen and stored at −80°C. Protein concentrations were determined using the Bio-Rad DC protein assay (Bio-Rad). For EMSA, we prepared a 43mer PCR product (nucleotides −1446 to −1404; 7), using a biotin-labeled primer (Table 1). Unlabeled competitor was prepared using similar but unlabeled primer pairs. Six micrograms of each nuclear extract or 1 μl of the TnT-product of the pRcCMV-hMafF plasmid was used for the binding reaction with 0.5 ng of biotin-labeled probe and the binding buffer described above, and electrophoresed in 5% PAGE/0.5× TBE buffer at 4°C. The gel was then blotted onto Biodyne B membrane (Pall) through 0.4 N NaOH. Biotinylated DNA was then detected using the Imaging high-chemilumi-kit (Toyobo) and exposed to Hyperfilm ECL film (Amersham-Pharmacia).

Enzymes and chemicals. Restriction enzymes were from New England Biolabs and Toyobo. Other chemicals were from local suppliers (Merk and Nakarai tesque) and were of analytical grade.

RESULTS AND DISCUSSION

Yeast one-hybrid screening with the $(US\text{-}2)_6$YM4271 strain, wherein the US-2 motif of the human OTR gene acted as target, yielded a total of 79 colonies which could be rescued in SD/-His/-Leu/+15 mM 3AT medium. As the β-galactosidase screening system using a $(US\text{-}2)_6$pLacZi vector (Clontech) gave a very high background (data not shown), another strategy was used to confirm the positive clones. All rescued plasmids were sequenced and checked by BLAST search of the international databases. Of these, 36 were either novel or related to DNA-binding proteins. HindIII-derived cassettes containing besides the expressed insert also the GAL4AD-cDNA sequence were then subcloned into the pRcCMV vector and fusion proteins synthesized using

FIG. 1. (A) Binding specificity of the GAL4AD-#1481 fusion protein to the OTR 5′ fragment (B1-1). Two microliters of *in vitro* transcription and translation (TnT) product, derived from the pRcCMV-GAL4AD-#1481 plasmid, was hybridized with radiolabeled B1-1 probe [nucleotides −1466 to −1375 bp of the human OTR gene (7)] and subjected to electrophoretic mobility shift assay (EMSA). For the supershift experiment, 1 μl of anti-GAL4 activation domain monoclonal antibody was preincubated with the binding reaction for 30 min. In the competition experiments, an excess of unlabeled DNA fragments was preincubated 10 min prior to the addition of radiolabeled B1-1 probe. Lane 1, 2 μl of TnT reaction mix without the template plasmid. Lane 2, TnT product of GAL4AD-#1481 fusion protein. Lane 3, as lane 2 plus anti GAL4 activation domain antibody. Lane 4, as lane 2 plus 20-fold molar excess of unlabeled A2 fragment. Lane 5, as lane 2 plus 20-fold molar excess of unlabeled B1 fragment. Lane 6, as lane 2 plus 2000-fold molar excess of unlabeled double stranded US-2A fragment. Lane 7, as lane 2 plus 2000-fold molar excess of unlabeled double stranded US-2B fragment. Lane 8, as lane 2 plus 40-fold molar excess of unlabeled unrelated fragment. The lower panel indicates the relation of each fragments to 5′-flanking of the human OTR gene. The exact location of A2 and B1 appeared in Kimura *et al.* (5). (B) Full-length hMafF cDNA sequence and deduced amino acid sequence. The asterisk indicates the stop codon. Italics indicate the sequence determined by 5′-RACE (25 bp) and 3′-RACE (650 bp). (C) Alignment with the human, chicken and mouse MafF, the members of the small Maf family. The alignment covers the full deduced amino acid sequences. Identical residues to hMafF are indicated by dots (.). The key residues for putative DNA binding domain, NxxYAxxCR, are boxed. The hydrophobic residues forming the heptad repeats of the leucine zipper domain are indicated by circles. The heptad repeat sequence LLLMLL is conserved in all members of the small Maf family including with chicken MafF and human h-Maf. (D) Molecular weight determination of the *in vitro* transcribed-translated (TnT) product derived from pRcCMV-hMafF. TnT reaction was performed using the pRcCMV-hMafF plasmid, containing full-length open reading frame of hMafF, in the presence of [^{35}S]-methionine. The radiolabeled product was subjected to denaturing SDS–PAGE (12% gel) with broad range protein size markers.

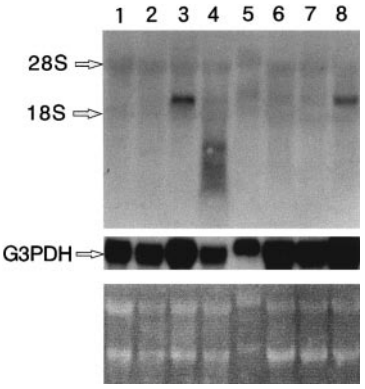

FIG. 2. Gene expression profile of hMafF. Twenty micrograms of total RNA from various human tissues was electrophoresed in 1% agarose/2.2 M formaldehyde/1× Mops gel and the gel was stained by ethidium bromide (lower panel). RNA was transferred to nylon membrane and hybridized with the radiolabeled *Sma*I–*Bam*HI fragment excised from pBS-#1481 plasmid. After high-stringency washing, the hMafF mRNA signal was detected by autoradiography (upper panel). The filter was then reprobed with for G3PDH mRNA as internal control (middle panel). Lane 1, nonpregnant myometrium. Lane 2, myometrium at 14 weeks gestation. Lane 3, myometrium after term delivery. Lane 4, placenta of 38 weeks gestation. Lane 5, choriodecidual membrane of 38 weeks gestation. Lane 6, liver, post mortem. Lane 7, spleen. Lane 8, kidney, post mortem.

the TnT combined transcription-translation system. Applying these protein products in an EMSA screening yielded 3 independent clones all showing obviously shifted signals, having a common sequence at their 5′-end (not shown). Binding specificity to the B1-1 fragment of the OTR gene promoter, which includes the US-2 motif, was assessed by supershift using an anti-GAL4 antibody or by competition experiment (Fig. 1A). The affinity of the protein to the DNA fragment appeared to be influenced by the length of the competitor fragment, since more than 100-fold molar excess of the US-2A fragment was required to compete the labeled B1-1 fragment, when compared to the unlabeled B1 fragment.

The longest of the identified positive cDNA clones was 1.8 kb. Additional sequence was then determined from 5′- and 3′-RACE reactions (Fig. 1B), which extended the sequence by a further 25 bp upstream and 650 bp downstream, respectively. Since no other open reading frame (ORF) further upstream can be identified, the ATG codon at nucleotide 147 is probably the true translation start site. The resulting ORF comprises 164 amino acids, has a predicted molecular weight of 17.8 kDa, and shows high homology to chicken (15) and murine MafF (16) (Fig. 1C) within the maf protooncogene family, named after V-maf from musculoaponeurotic fibrosarcoma of chicken (17). The US-2 binding protein is thus probably the human homologue of MafF (hMafF). The amino acid region critical for DNA binding is considered to be the NxxYAxxCR motif, comparable to the NxxAAxxSR motif for GCN4 (18) and the NxxAAxxCR motif of Jun-Fos heterodimers (19). The hydrophobic residues forming the heptad repeats of the leucine zipper domain are also conserved, as in all other members of the small Maf family (Fig. 1C). Also like the other small Maf proteins, hMafF lacks a distinct N-terminal transactivating domain. *In vitro* translated hMafF protein shows a molecular weight in denaturing PAGE of 18 kDa (Fig. 1D), in accord with the size predicted from the cDNA.

From the BLAST database search, the 3′-UTR segment of the hMafF cDNA shows a high homology score to several different BAC clones from diverse human chromosomes, suggesting that this region is duplicated from an ancestral gene and is not unique to hMafF. In contrast, the coding region of the hMafF cDNA shows homology only to murine and chicken MafF cDNA. Thus, to avoid cross-hybridization with other related sequences, northern hybridization was performed using the unique BamHI-SmaI fragment (nucleotides 156 to 578) from the coding region of the cDNA as a probe (Fig. 2). Indeed, Northern blotting using a probe from the 3′-UTR indicated a ubiquitous expression pattern even after high stringency washing (not shown), suggesting that at least some of the sequences found in the database may be transcribed as general housekeeping genes. Using the probe specific for hMafF, a strong 2.6-kb specific signal is seen in RNA from term myometrium and from kidney. In the placenta, weak signals of smaller (1.0 and 0.8 kb) sizes are also observed

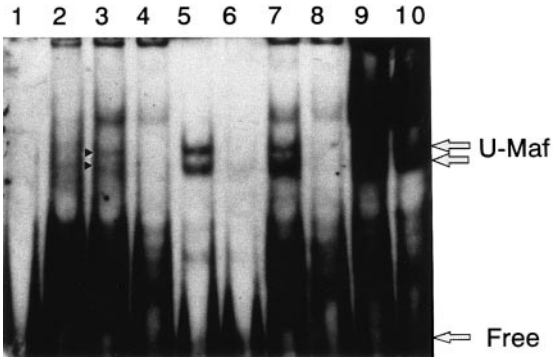

FIG. 3. hMafF protein in various human tissues determined by EMSA. Six micrograms of nuclear proteins extracted from various human tissues or 0.5 μl of the TnT product of the pRcCMV-hMafF plasmid was subjected to EMSA. The biotin-labeled 43-mer probe containing the US-2 element was generated by PCR, and visualized by chemiluminescence. Signals showing the same mobility shift as that for the hMafF TnT product are indicated by arrowheads. Lane 1, free probe. Lane 2, 6 μg of nuclear extract from non-pregnant myometrium. Lane 3, 6 μg nuclear extract from term myometrium. Lane 4, as lane 3 plus 100-fold molar excess of unlabeled 43mer probe. Lane 5, 0.5 μl of hMafF TnT product. Lane 6, as lane 5 plus 100-fold molar excess of unlabeled 43mer probe. Lane 7, 6 μg of term myometrial nuclear protein plus 0.5 μl of hMafF TnT product. Lane 8, as lane 7 plus 100-fold molar excess of unlabeled 43mer probe. Lane 9, 6 μg of liver nuclear extract. Lane 10, as lane 9 plus 100-fold molar excess of unlabeled 43mer probe.

(Fig. 2). The integrity of mRNA in each tissue was checked by rehybridization of the blot with a G3PDH probe as well as by ethidium bromide staining of the ribosomal RNA. Also for chicken and murine MafF, expression was found to be highly tissue-specific. Maximum expression was observed in the ovary, and low level of expression was detected in the brain, heart and mesenterium of chicken tissues (15), while murine MafF was highly expressed only in the lung (16).

Using EMSA with the 43mer fragment of the OTR gene including the US-2 motif as probe (Fig. 3), the *in vitro* transcribed-translated (TnT) hMafF protein could be detected as two shifted bands, possibly representing monomeric and dimeric complexes (lane 5). There is a weak but significant signal due to hMafF using nuclear extracts from term myometrium (lane 3), but not with those from non-pregnant uterus (lane 2). Although the probe is relatively short, there appears nevertheless to be a high degree of unspecific binding, often of stronger intensity, for example in the liver nuclear extract (lane 9). Competition with a 100-fold molar excess of the unlabeled probe failed to eliminate these unspecific signals (lane 10), whereas this amount of competitor was sufficient to displace both the signal from term myometrium as well as the TnT hMafF protein (lanes 4 and 6). Small maf proteins are believed to form heterodimers with NF-E2 (p45), Fos, Nrf 1, Nrf 2 (20), Bach1 (21) and Bach2 (22) to bind the Maf recognition element (MARE). To determine whether hMafF might binds to the US-2 element as a heterodimer, we mixed nuclear extract from term myometrium with the TnT-derived hMafF protein. However, no extra band can be detected when compared with the original term nuclear factor, although the intensity of the second band was increased (lane 7). It is not clear at this stage if there might not be some other protein(s) interacting with hMafF *in vivo.*

Since we have shown that hMafF can bind specifically to the OTR gene *in vitro*, the expression of this protein in term myometrium strongly suggests that it also interacts with the activated OTR gene *in vivo.* Lacking the N-terminal transactivation domain found in the large maf family, but absent in the small maf family, hMafF is probably unable by itself to activate a target gene. Indeed, when we cotransfected the pRcCMV-hMafF expression plasmid, together with an OTR gene promoter-luciferase construct, with or without the US-2 recognition motif, into MCF-7 cells, we unsurprisingly failed to observe any significant alteration of the basal luciferase activity (not shown). The sequence of the US-2 element shows some similarity to the AP-1 and NF-E2 binding motifs. Thus, like the small mafs, MafK and MafG, hMafF might function by interacting with other transactivating factors directly, or by modulating the binding of other nuclear factors which interact with the OTR gene close to the US-2 element. Although, it has recently been shown that the c-Fos/c-Jun complexes can bind to an AP-1 site and activate human OTR gene transcription in a breast cancer cell line (23), the absolute levels of activation achieved were relatively low, and the binding site implicated in this action was close to the transcription start site and not near the US-2 element. It is still completely unknown whether molecules, such as NF-E2 (p45), Nrf1, Nrf2, Bach1 or Bach2, which interact with other small mafs, can interact with hMafF or bind the OTR gene promoter near the US-2 motif and thereby regulate OTR gene transcription. Also, the significance of hMafF expression in the human kidney, where the OTR gene is not highly expressed, is not known. Recently, mice have been reported in which the mouse mafF gene has been ablated (16). Although, uterine expression of mouse MafF has not been investigated, the mutant mice showed no apparent defect in reproduction nor parturition. However, many genes known to be involved in parturition, fail to show a phenotype in such ablation experiments (24).

In conclusion, we have cloned a human homologue of MafF (hMafF) which specifically interacts with the upstream promoter region of the human OTR gene. The cellular and temporal pattern of expression of hMafF *in vivo* correlates very closely with the massive upregulation of the OTR gene at term of pregnancy. From its structure, hMafF is more likely to be a mediator of specificity rather than an activator, though its precise function needs to be further characterized. We would predict that hMafF is actively involved *in vivo* in the upregulation of the OTR at term of pregnancy, and probably in other associated aspects of the parturition process.

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